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HPRT YEAST ARTIFICIAL CHROMOSOME TRANSFER INTO HUMAN CELLS BY FOUR METHODS AND AN INVOLVEMENT OF HOMOLOGOUS RECOMBINATION

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SUMMARY: The transfer of a yeast artificial chromosome (YAC) into mouse and human cell lines was effected by four methods, and the efficiency and integrity of the incorporated YAC DNA were compared. A 500 kb YAC containing the human hypoxanthine-guanine phosphoribosyl transferase (HPRT) gene was transferred more efficiently by polyethylene glycol-mediated fusion than by lipofection, electrofusion, or electroporation. Southern blot analysis demonstrated that PEG fusion lines yielded fragments of the size of the original YAC clone, whereas lipofection and electroporation did not. Two of 53 fusion lines showed 6-thioguanine resistance and confirmatory disruption of the HPRT gene in the YAC DNA, suggesting that the YAC DNA was integrated by homologous recombination with the endogenous HPRT gene region.

Yeast artificial chromosomes (YACs) (1) have been well established as tools to clone genes (2, 3) and to make physical maps (4-6). We have recently improved the construction of the YAC-library by partial fill-in method (7). From the earliest discussions, however (8), the potential advantages of YACs for the analysis of mammalian gene expression and genetic recombination have also been clear. Because of their gigantic sizes, YACs might provide a route to study functional complementation in expression cloning systems, and even to introduce genes to correct aberrant variants. Critical to such proposals is the optimization of methods to introduce YAC clones into mammalian cells and to promote homologous recombination. Four methods have been used to introduce YAC clones into mammalian cells; polyethylene glycol (PEG)-mediated spheroplast fusion (9-11), calcium phosphate transfection (12, 13), lipofection (14, 15) and microinjection (16). We have compared several of these methods and

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two additional ones, electrofusion and electroporation, in respect to efficiency of transfer of a YAC and features of the structure of the transferred DNA.

Gene targeting studies have shown that the rate of homologous recombination via random integration increases as the size of DNA fragments introduced into cells increased toward 40 kb (17). Such a trend might be accentuated with the much larger YAC DNAs. However, studies thus far have generally transferred human YAC DNA into rodent cells, which would preclude much of the high homology that is necessary for homologous recombination. To see if recombination would be favored with homologous DNA, we assessed the state of YAC DNA integrated in a human cell line. The results indicate that PEG fusion as well as calcium phosphate-mediated transfer (13) favors the intactness of the transferred DNA more than the other methods; and the homologous recombination in human cells indeed occurs, reaching levels of 4 % or more of the transformed colonies.

MATERIALS AND METHODS

YAC clone, cell lines and PCR primers : A 500 kb YAC clone containing the human hypoxantihine-guanine phosphoribosyl transferase (HPRT) gene region has been isolated from a human Xq chromosome specific YAC library (4, 18). This clone contains the distal portion of the HPRT gene from exon 4. Yeast transformation was performed according to Burgers & Percival (19). Mouse L cells and human Hel cells (20) were kindly provided from Dr. Y. Kaneda of Osaka University. Both cell lines were cultured in MEM (GIBCO) containing 10 % fetal calf serum. PCR primer pairs (ACCAAGTGCCTTGTCTGTAGTGTC and GAACTGCTGACAAAGATTCACTG) for amplification of the HPRT gene were kindly provided form Dr. K. Tatsumi at Kyoto University.

Transfer of YAC into mammalian cell : PEG-spheroplast fusion was performed essentially as described by Pavan et al. (10). A concentration of 80 mg/ml for Zymolyase 100T (Seikagaku Kogyo Co., Tokyo) and 10^8 cells for yeast yielded best results. Total yeast DNA, including YAC DNA for lipofection and electroporation, was prepared according to Olson et al. (21). Pulsed-field gel analysis showed that DNA of at least less than 690 kb did not seem to be degraded. Lipofection and electroporation were performed as described previously (15, 14 and 22). Spheroplasts for electrofusion were prepared as for PEG fusion except they were suspended with 0.7 M sorbitol, 0.2 mM Tris-HCl (pH 7.2), 0.1 mM $CaCl_2$, and 0.1 mM $MgCl_2$. Mammalian cell was harvested, washed with phosphate buffered saline, and resuspended in the same solution. Electrofusion was carried out according to Noda et al. (23) with 200V/cm of AC field applied before (30 sec), between, and 30 sec after the DC pulses. Three 2.1 kv/cm, 60μ sec pulses at interval of 1 sec were used.

DNA preparation and Southern blot analysis : DNA solutions were made as previously described (24, 25), as was DNA in agarose blocks (26, 27). Pulsed-field gel electrophoresis (PFGE) was carried out according to Chu et al. (28). Transfer of DNA to Nylon membrane (BiodyneB, Paul) in alkaline solution and hybridization was performed as described by Sambrook et al. (24). For competition hybridization in Fig. 3C, total yeast DNA containing HPRT YAC DNA was labeled by nick translation and preannealed with sheared total human DNA extracted from human Hel cell line and sheared total yeast DNA extracted from yeast strain AB1380, each at a concentration of 15 mg/200 ml in 2 x standard saline citrate (18).

RESULTS

Modification of YAC clones carrying the human HPRT gene

Conditions to transfer the YAC clone into mammalian cells were established, and the fate of the introduced YAC was then assessed. For these purposes, we introduced the neomycin

(Neo) gene as a selectable marker into the HPRT gene (Fig. 1A). If the introduced YAC DNA were integrated by homologous recombination, cells would become HPRT$^-$, easily recongnized by their acquired resistance to 6-thioguanine. Disruption of the HPRT gene on the YAC and retrofitting the neomycin resistant selectable marker to the YAC were done as follows (Fig. 1, A). [1] A two-kb DNA fragment containing the HPRT exon 7, 8 and 9 was amplified by PCR using primer pairs described in MATERIALS AND METHODS and isolated. [2] 0.2 kb NspI

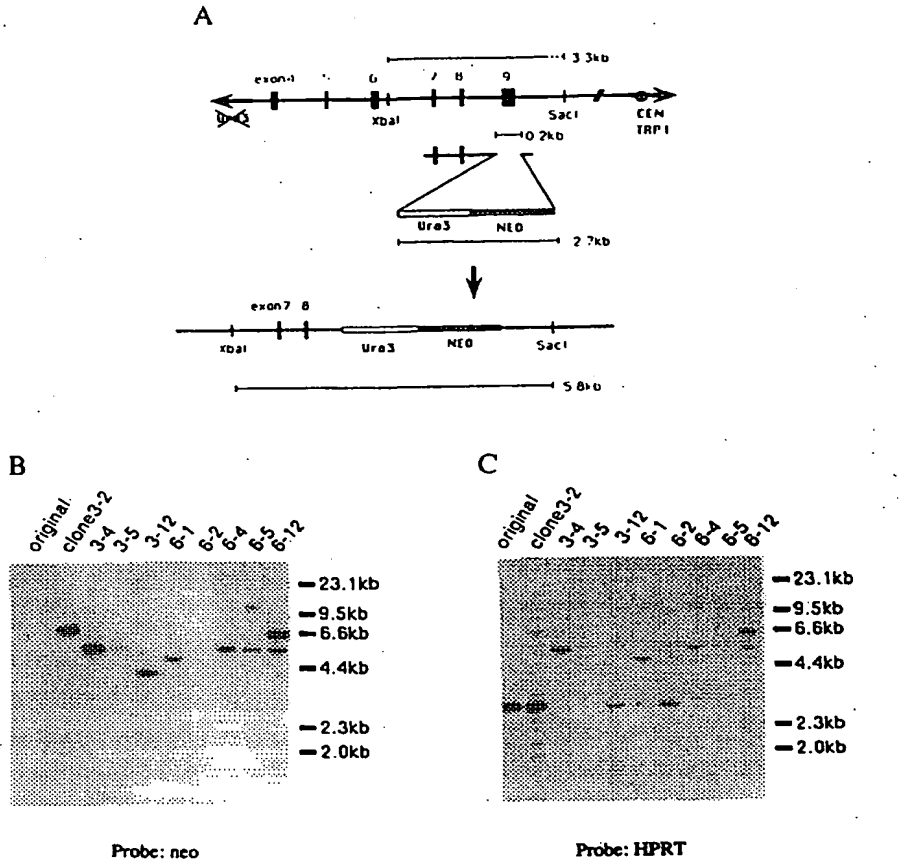

Figure 1. Integration of the Neo gene into the YAC carrying human hprt region.
(A) Schematic representation of integration of Neo gene. (Top) HPRT region of original YAC. Exon 4-9 of HPRT gene and relevant restriction sites are shown. Ura3 gene on the right arm of the YAC has been disrupted by inserting Lys2 gene. (Middle) Ura3 and Neo gene have been replaced by exon 9 of HPRT on the fragment which has been amplified by PCR. (Bottom) Part of the modified YAC. Size of each region is indicated in kb. (B) Southern blot analysis of the modified YAC. DNA was extracted from each yeast clone carrying original HPRT YAC or modified YAC (clone 3-2 etc.) and electrophoresed after digestion by XbaI-SacI. DNA was transferred to Nylon membrane and hybridized with Neo probe. (C) Same filter as (B) was reprobed with HPRT probe.

fragment containing exon 9 of this 2 kb fragment was replaced with Ura3 gene and Neo gene in vitro. [3] The DNA fragment constructed in the step 2 was transformed into yeast cells carrying the HPRT YAC clone with the Ura3 gene on the vector arm disrupted by a Lys2 fragment beforehand (12). Transformants were selected for growth without uracil, and were expected to contain YAC DNA in which exon 9 is replaced by Ura3-Neo DNA by homologous recombination at the exon 7-9 region.

Recombination was confirmed by Southern blot analysis as shown in Fig. 1 B and C. XbaI-SacI double digestion of human genomic DNA generates a 3.3 kb fragments, but a 5.8 kb fragments should rather be generated by equivalent restriction digestion of recombinants, as shown in Fig. 1A. Clones 3-4, 3-5, 6-4 and 6-5 showed the expected 5.8 kb fragment indicative of recombination, and the 5.8 kb species also hybridized to the Neo probe (Fig. 1B and C), consistent with placement of the Ura3-Neo fragment at the appropriate position. The overall effeciency of correct retrofitting was 40 % (4 out of 9 clones).

Comparison of efficiency of YAC transfer by four different methods

The frequency of introduction of YAC clones and the integrity of YAC DNA transferred were assessed. Frequency of YAC transfer was estimated by scoring the number of G418-resistant ($G418^r$) clones obtained by each method. Eighteen to 53 $G418^r$ clones were obtained by PEG fusion depending on the condition used. On the other hand, 41, 3 to 10, and 2 to 5 $G418^r$ clones were obtained by electroporation, lipofection, and electrofusion, respectively. It is difficult to compare these frequencies directly, since extracted DNA was used for electroporation and lipofection, whereas intact cells were used in the fusion method. However, in typical experiments, PEG fusion pragmatically yielded the highest frequency per mammalian cell recipient in standard conditions.

Assessment of integrity of the YAC DNA transferred

We initially used mouse L cell line as the recipient for YAC, since the state of the human DNA in stable transformants is relatively easy to analyse. DNA was extracted from the fusion lines or lipofectants and analyzed by Southern blotting as shown in Fig. 2. A fragment of about 10 kb, the same size as in the original YAC clone, was detected in the PEG fusion line but not in the lipofectant (Fig. 2B). To see if gross rearrangement of transferred human DNA had occurred or not, DNA extracted from the fusion line was digested by MluI and analysed by PFGE. A 90 kb fragment seen in the original YAC clone hybridized with Neo gene probe in all fusion lines tested indicating that this 90 kb portion [including the HPRT gene (Fig. 2A)] has been transferred intact. Fragments about the same size as the original YAC clone (270 kb and 90 kb) were also detected in the fusion line with an Alu probe (data not shown). No band was detected outside of the compression zone in the lipofectant, suggesting that gross rearrangemnents of the introduced YAC DNA had likely occurred. PEG fusion thus gave more transformants and likely preserved the DNA more intact than did lopofection. YAC DNA transferred by electroporation seemed fragmented or rearranged, but no bulk rearrangement was observed after transfer by electrofusion (data not shown).

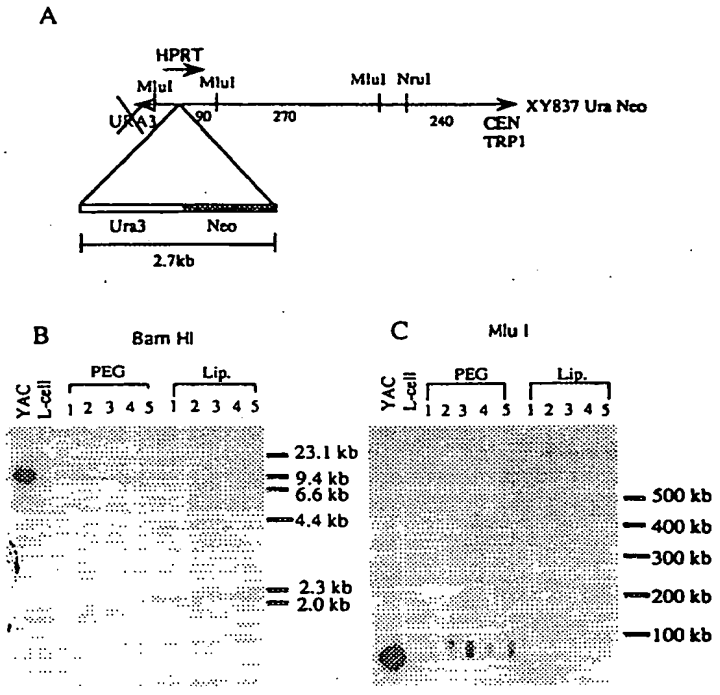

Figure 2. Transfer of the modified YAC clone carrying HPRT region into L cell line. (A) Diagram of the modified YAC containing the HPRT region. MluI and NruI sites are indicated. Size of each restriction fragment is shown in kb. (B) Southern blot analysis of PEG fusion lines (PEG) and Lipofectants (Lip). "YAC" indicates original YAC to be introduced. Neo probe was used for hybridization. (C) PFGE analysis of PEG fusion lines (PEG) and Lipofectants (Lip). High molecular weight DNA was prepared in agarose blocks as described in MATERIALS AND METHODS and electrophoresed after digestion by MluI. DNA was transferred to Nylon membrane and hybridized to a Neo probe.

Next, human Hel cell line was used as the recipient for YAC transfer to assess for homologous recombination. DNA was then extracted, run on a conventional agarose gel, and analyzed by Southern blotting with whole YAC DNA as a probe. Several fragments of the same size as in the YAC DNA were detected in some fusion lines (data not shown) suggesting again that YAC DNA has been transferred without major rearrangement in some fusion lines.

Targeted integration of YAC DNA into the homologous region of host chromosome

In an additional test, clones transferred into the Hel cell line were assessed for possible homologous recombination. Because the YAC clone we used carries part of the HPRT gene disrupted by insertion of Neo gene at exon 9 (Fig. 1), a fusion cell line in which YAC DNA is integrated by homologous recombination should be HPRT deficient and should show a 6-thioguanine resistant phenotype. In fact, two of 53 fusion lines were resistant to 6-thioguanine.

Supporting evidence for homologous integration was obtained by Southern analysis. Exon 9 of human HPRT gene included in a 50 kb ClaI fragment (Fig. 3A). The 50 kb ClaI fragment should be detected by a neo probe if the modified YAC HPRT clone has been integrated by homologous recombination, however, the size of fragment detected by a Neo probe should be of any size when the YAC clone has been randomly integrated. As expected,

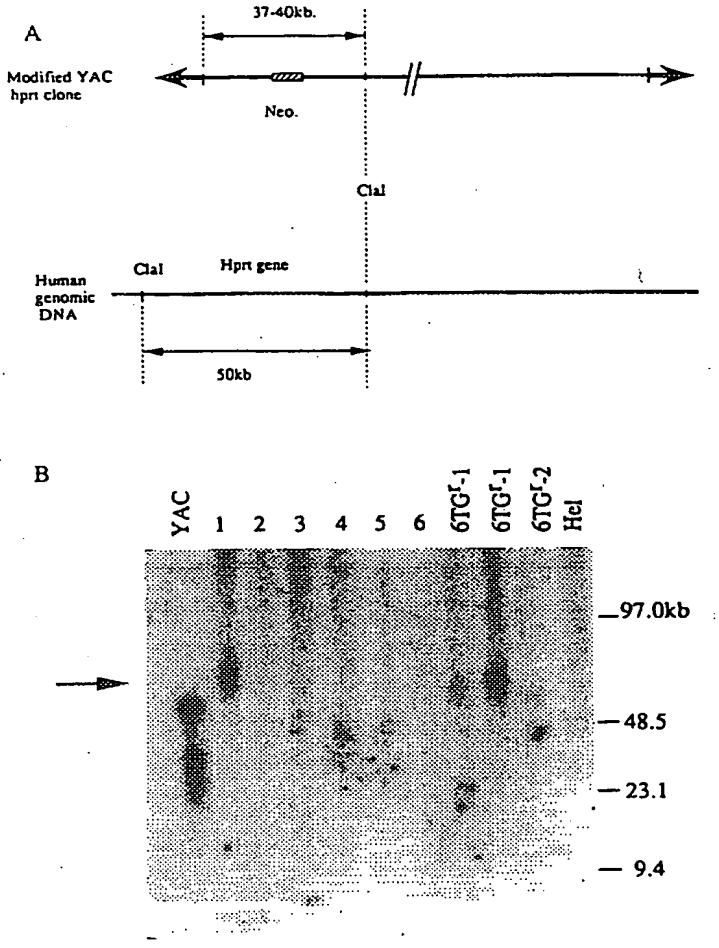

Figure 3. PFGE analysis of the modified HPRT YAC DNA in PEG fusion-Hel cell lines. (A) Physical map of HPRT region of the modified HPRT YAC (Top) and human genomic DNA (Bottom). (B) DNAs were extracted from yeast carrying the modified YAC (YAC), PEG fusion lines which were 6-thioguanine sensitive (1-6), PEG fusion lines which were 6-thioguanine resistant (6TG^r-1, 6TG^r-2) and Hel cell line, and PFGE was performed after digestion with ClaI. Then, Southern blot hybridization was performed with Neo probe. Arrow indicates expected size of ClaI fragment which is hybridized with Neo probe, when the YAC has been integrated into the endogenous HPRT region by homologous recombination.

digestion of DNA from 6-thioguanine resistant fusion lines with ClaI generated a 50 kb-Neo specific fragment (Fig. 3B). On the other hand, when DNA was extracted from 6-thioguanine sensitive lines, ClaI fragment of various sizes hybridized with the Neo probe (Fig. 3B). These results were also confirmed by Southern analysis using HpaI enzyme (data not shown).

DISCUSSION

The PEG fusion method generated consistently productive yields of cell lines, and without gross rearrangement. Presumably this is at least partially because methods like lipofection and electroporation use DNA extracted from cells, and involve a high risk of shearing. However, indications of relative intactness have been seen with calcium phosphate-mediated transfer as well (13); and rearrangement was detected by Southern blot analysis of DNA even in some lines derived by PEG fusion. Thus care should clearly be taken in choosing any fusion line for further study.

The frequency of recovery of transformed clones is consistent with results of Pachnis et al. (9), who used a 450 kb anonymous YAC clone and Neo selection in mouse L cells, and by Huxley et al. (11), who used a 680 kb HPRT YAC clone and direct HAT selection in an L cell variant. Thus PEG fusion works comparably with several selection methods in multiple cell lines, extended here to the use of human cells as a recipient.

At least two homologous integration events among 53 transformants is a promising and very high rate of occurrence. Thomas & Capecchi (29) found that 1/1000 transfectants were generated by homologous integration when ES cells were the recipient for plasmid DNA containing a 9 kb mouse HPRT gene. The higher rate observed here might result because homologous recombination occurs especially efficiently in the HeI cell line used, or because random integration is inhibited in the cells; but the simplest interpretation of the results is that the large size of incoming YAC DNA favored homologous recombination. Other possibilities of placement of the YAC or complex chromatin structure remain open; but it seems likely that the much greater extent of homologous tracts in larger incoming DNA fragments is the critical factor (see Introduction). It is interesting to see the effect of the length of the YAC DNA on the frequency of homologous recombination. In any case, the results strongly support the possible utility of YACs as an efficient tool for gene targeting.

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